Interrelationship between renin-angiotensin-aldosterone system and oxidative stress in chronic heart failure patients with or without renal impairment

Marta Reina-Couto [a,b,c], Joana Afonso [a,b], Jorge Carvalho [a], Luís Morgado [a,c], Fernanda Aparecida Ronchi [d], Ana Paula de Oliveira Leite [d], Claudia Camila Dias´ [e,f], Dulce Elena Casarini [d], Paulo Bettencourt [c,g], Antonio Albino-Teixeira´ [a,b], Manuela Morato [h,1,**], Teresa Sousa [a,b,*,1]

[a] Departamento de Biomedicina – Unidade de Farmacologia e Terapˆeutica, Faculdade de Medicina, Universidade do Porto, Rua Dr. Placido da Costa, S/N, Edifício ´ Poente, Piso 3, 4200-450 Porto, Portugal

[b] Centro de Investigaçao Farmacol˜ ogica e Inovaç´ ao Medicamentosa, Universidade do Porto (MedInUP), Alameda Professor Hern˜ ani Monteiro, 4200-319, Porto, Portugal ˆ [c] Centro Hospitalar Universitario de S´ ao Jo˜ ao, Alameda Professor Hern˜ ani Monteiro, 4200-319 Porto, Portugal ˆ

[d] Departamento de Medicina, Disciplina de Nefrologia, Escola Paulista de Medicina, Universidade Federal de Sao Paulo, Rua Botucatu, 740, Vila Clementino, 04023900, ˜ Sao Paulo, SP, Brazil ˜

[e] Departamento de Medicina da Comunidade, Informaçao e Decis˜ ao em Saúde, Faculdade de Medicina, Universidade do Porto, Rua Dr. Pf˜ acido da Costa, S/N, Edifício ´ Nascente, Piso 2, 4200-450 Porto, Portugal

[f] CINTESIS - Centro de Investigaçao em Tecnologias e Serviços de Saúde, Porto, Portugal ˜

[g] Departamento de Medicina, Faculdade de Medicina, Universidade do Porto, Alameda Professor Hernani Monteiro, 4200-319, Porto, Portugal ˆ

[h] Departamento de Ciˆencias do Medicamento, Laboratorio de Farmacologia, Faculdade de Farm´ acia, Universidade do Porto ´ & LAQV@REQUIMTE, Rua Jorge Viterbo Ferreira nº 228, 4050-313, Porto, Portugal

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ABSTRACT

We investigated oxidative stress and RAAS biomarkers, as well as their association, in chronic heart failure (CHF) patients on optimized medical therapy, stratified by disease severity or by renal function. Since vitamin D has been shown to attenuate RAAS activation and oxidative stress, we further evaluated the relationship between vitamin D, RAAS and oxidative stress in CHF patients with or without renal impairment. Sixty CHF outpatients were included and stratified by disease severity or by renal function. We quantified urinary hydrogen peroxide, plasma and urinary isoprostanes, plasma total antioxidant status, urinary angiotensinogen (intrarenal RAAS activation biomarker) and plasma angiotensinogen, plasma renin and aldosterone concentration, serum angiotensin-converting enzyme (ACE) activity, plasma angiotensin peptides, and serum total 25-hydroxyvitamin D (S-total 25(OH)D). Severe CHF patients had higher urinary isoprostanes ($p = 0.002$) and lower S-total 25(OH)D ($p = 0.006$) compared to mild-to-moderate patients, but no differences were observed for other redox or RAAS biomarkers. Patients with impaired renal function (iRF) had higher urinary angiotensinogen ($p = 0.003$) and lower S-total 25(OH)D ($p = 0.028$) compared to those with normal renal function (nRF), while no differences were observed for the remaining RAAS and redox parameters. Several positive correlations between oxidative stress and RAAS biomarkers were detected in iRF patients, while in patients with nRF these correlations were primarily inverse. In CHF-iRF patients, S-25(OD)D was

Abbreviations: ACE, angiotensin-converting enzyme; ACEi, angiotensin-converting enzyme inhibitors; AGT, angiotensinogen; AKI, acute kidney injury; Ang I, angiotensin I; Ang II, angiotensin II; BHT, butylated hydroxy toluene; BNP, B-type natriuretic peptide; CHF, chronic heart failure; CRS, cardiorenal syndrome; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; FAPGG, N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine; H_2O_2, hydrogen peroxide; HF, heart failure; iRF, impaired renal function; LVSD, left ventricular systolic dysfunction; MR, mineralocorticoid receptor; nRF, normal renal function; NYHA, New York Heart Association; S-ACE, serum angiotensin-converting enzyme activity; P-AGT, plasma angiotensinogen; P-Ang I, plasma angiotensin I; P-Ang II, plasma angiotensin II; P-Ang(1-7), plasma angiotensin 1-7; P-Isop, plasma isoprostanes; P-renin, plasma renin concentration; P-TAS, plasma total antioxidant status; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; P-Aldost, plasma aldosterone concentration; SEM, standard error of the mean; S-total 25 (OH)D, serum total hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-H_2O_2, urinary hydrogen peroxide; U-Isop, urinary isoprostanes.

* Corresponding author at: Departamento de Biomedicina – Unidade de Farmacologia e Terapˆeutica, Faculdade de Medicina, Universidade do Porto, Rua Dr. Placido da Costa, S/N, Edifício Poente, Piso 3, 4200-450 Porto, Portugal. ´

** Corresponding author at: Departamento de Ciencias do Medicamento, Laboratˆ orio de Farmacologia, Faculdade de Farm´ acia, Universidade do Porto, Rua Jorge ´ Viterbo Ferreira nº 228, 4050-313, Porto, Portugal.

E-mail addresses: mmorato@ff.up.pt (M. Morato), tsousa@med.up.pt (T. Sousa).

[1] These authors contributed equally to the supervision and scientific revision of this paper.

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inversely associated with urinary isoprostanes, which in turn were positively associated with plasma angiotensinogen and serum ACE. In conclusion, CHF patients with renal function impairment have increased intrarenal RAAS activation and lower vitamin D values and might benefit from the combination of RAAS blockers with vitamin D and/or antioxidants.

1. Introduction

The coexistence of kidney and heart failure (HF) is increasing and represents a major clinical challenge that is nowadays defined as the cardiorenal syndrome (CRS) [1]. Its pathogenesis is complex and yet not well understood, but is associated with adverse prognosis [2].

It is well established that systemic and renal oxidative stress can contribute to CRS pathology, particularly in CRS type 2 [3,4]. Among reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) appears to be particularly relevant in the context of CRS as it is generated by various enzymes in the heart and kidney, exerts both protective and deleterious effects in the heart and contributes to renal injury [5–11].

Activation of the renin-angiotensin-aldosterone system (RAAS) is also widely recognized as an important neurohumoral mechanism in HF, being involved in both cardiac and renal dysfunction [12,13]. Moreover, independently from the systemic RAAS, activation of local tissue system also plays a role in the pathophysiology of renal disease and HF [14,15]. In this context, the urinary excretion of angiotensinogen (AGT) appears to be particularly relevant as it is considered an index of intrarenal RAAS activity and has been associated with renal dysfunction and arterial hypertension [16,17]. Furthermore, it has also been suggested as a prognostic marker in renal diseases and in acute decompensated HF [18–20].

RAAS and oxidative stress are known to be interrelated. It has been extensively demonstrated that angiotensin II (Ang II) stimulates ROS generation by NADPH oxidases and other prooxidant enzymes, thus contributing to cardiovascular and renal dysfunction [21–24]. Recent evidence supports that chronic RAAS activation can increase oxidative stress, leading to renal injury and to a vicious cycle of more sodium and water retention in CRS type 2 [3]. However, although angiotensin-converting enzyme inhibitors (ACEi) are a first-line treatment in HF [25], even in the presence of renal dysfunction [26], they seem to have little benefit in the setting of existing CRS type 2 [27]. Interestingly, accumulating evidence indicates that ROS also regulate RAAS activation [7,23,28], although evidence comes mostly from experimental studies concerning the kidney, where ROS were shown to increase AGT, renin, angiotensin-converting enzyme (ACE), Ang II, mineralocorticoid receptor (MR) and aldosterone [28]. Thus, it is possible that the lack of significant benefits from ACEi therapy in the setting of CRS type2 [27] results from its inability to effectively target the self-perpetuating cycle of RAAS-oxidative stress-renal dysfunction. Of note, there is evidence suggesting that renal impairment might be a major factor determining the relevance of therapies targeting oxidative stress in HF. While antioxidant treatment was shown to reduce cardiovascular morbidity and mortality in patients with end-stage renal disease, it did not exert a significant impact in patients at high-risk for cardiovascular events but with normal or slightly altered renal function [29,30].

Few studies evaluated both oxidative stress and RAAS activation biomarkers in patients with chronic HF (CHF) under optimized medical therapy [31,32]. This is probably due to the anticipated difficulties in interpreting results in patients receiving a combination of several medications that may interfere with both pathways [33].

We aimed to characterize, comprehensively, oxidative stress and RAAS in patients with CHF under optimized medical therapy, stratified by disease severity or by renal function. Newly, we looked at H_2O_2, AGT and several angiotensin peptides, which have been scarcely explored in human CHF and CRS, although we also measured commonly used markers of oxidative stress (e.g. isoprostanes, total antioxidant status) and RAAS (e.g. plasma renin, ACE activity, aldosterone) [34–39]. Since renal disease is associated with insufficiency or deficiency of vitamin D [40,41], which has been shown to exert protective effects against oxidative stress and RAAS over-activation [42–45], we also investigated the relationship between vitamin D status, oxidative stress and RAAS parameters in CHF patients with or without renal dysfunction.

2. Methods

2.1. Study population

Outpatients (n = 60) with an established HF diagnosis (New York Heart Association (NYHA) classes I to IV) for at least 2 years, with left ventricular systolic dysfunction (LVSD) assessed by echocardiography and optimized medical therapy, were recruited from the HF clinic of Centro Hospitalar Sao Jo˜ ao, Porto, Portugal. We excluded patients with ˜ history of acute coronary syndrome, any hospital admission, therapeutic adjustment or infection, in the previous 3 months. All eligible patients provided written informed consent to participate in the study. This study was conducted in accordance with the Guidelines for Good Clinical Practice and the 1975 Declaration of Helsinki after approval by the institution's Ethics Committee.

2.2. Data collection

All patients were asked to collect a 24-h urine sample (8 AM to 8 AM the next morning) along the day before the visit to the HF clinic of Centro Hospitalar Sao Jo˜ ao. On the day of the visit, the patients brought ˜ the 24h-urine samples to the clinic and fasting venous blood samples were collected between 8 AM and 11 AM. A physical examination was performed to all patients, and a record of demographic data, comorbidities, and medication in use was completed. To prevent possible bias concerning the clinical evaluation and NYHA class assignment, the same physician examined all the patients. However, we cannot exclude bias from patients' subjective self-assessment of symptoms and physical limitations.

CHF patients were stratified by disease severity or by the presence or absence of CRS defined here as impaired renal function (iRF) (estimated glomerular filtration rate, eGFR < 60 mL/min per 1.73 m^2) or normal renal function (nRF) (eGFR $>$ 60 mL/min per 1.73 m^2), respectively. [46]

2.3. Laboratory procedures

All the routine laboratory analyses were performed at the Clinical Pathology Department of CHSJ. Plasma B-type natriuretic peptide (BNP) and troponin I were quantified by chemiluminescent microparticle immunoassays using an Abbot® Architect i2000 automated analyser (Abbott® Diagnostics, Lake Forest, IL, USA). An Olympus AU5400,

Beckman Coulter® automated clinical chemistry analyser (Olympus, Hamburg, Germany) was used for the quantification of serum urea concentration by a kinetic urease/glutamate dehydrogenase method, plasma and urine creatinine by the colorimetric Jaffe method and urinary protein by the pyrogallol red method. Urinary sodium was measured by an ion-selective electrode method. Plasma renin concentration (P-renin) and plasma aldosterone concentration (P-aldosterone) were measured by a radioimmunoassay and serum ACE activity (S-ACE) was quantified in an Olympus AU5400, Beckman Coulter® automated clinical chemistry analyser (Olympus, Hamburg, Germany) by a spectrophotometric method using N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) as substrate (Bühlmann®, Bühlmann Laboratories AG, Schonenbuch, Switzerland). Vitamin D status was ¨ evaluated by measuring serum total 25-hydroxyvitamin D (S-total 25 (OH)D) [47] by an electrochemiluminescent immunoassay using a Cobas E411 automated analyser and the Elecsys® Vitamin D total kit (Roche Diagnostics GmbH, Mannheim, Germany). All the samples used for the determination of other RAAS (AGT and angiotensin peptides) or oxidative stress biomarkers were processed at the Department of Biomedicine – Unit of Pharmacology and Therapeutics, Faculty of Medicine of Porto, Portugal, and stored at -80 °C until assayed.

2.4. Quantification of AGT and angiotensin peptides

AGT was quantified in plasma (P-AGT) and spot urine samples (U- AGT) by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Human Total Angiotensinogen Assay Kit, Immuno- Biological Laboratories Co., Gunma, Japan) accordingly to the protocol provided by the manufacturer.

Angiotensin I (Ang I), angiotensin II (Ang II) and angiotensin 1-7 (Ang 1-7) peptides were extracted from plasma samples using Sep- Pack-C18 columns (Discovery® DSC-Ph SPE, Supelco by Sigma- Aldrich/Merck, Sintra, Portugal) and then, the plasma values of Ang I (P-Ang I), Ang II (P-Ang II) and Ang 1-7 (P-Ang 1-7) were quantified by reverse phase high-performance liquid chromatography with UV detection [48]. Peaks of interest were identified by retention time of peptide standards and the results expressed in pg/mL.

2.5. Quantification of oxidative stress biomarkers

H_2O_2 was quantified in 24h-urine samples (U-H_2O_2) by a microplate fluorimetric assay (Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit; Molecular Probes, Invitrogen, Thermo Fisher Scientific, Massachusetts, USA). Urinary isoprostanes (U-Isop) were quantified in 24h-urine samples by a competitive enzyme immunoassay (Urinary Isoprostane ELISA Kit; Oxford Biomedical Research® Inc., Oxford, MI, USA) in non- extracted urine containing the preservative butylated hydroxy toluene (BHT, 0.005 %, w/v) added before storage, and incubated with β-glucuronidase before the assay. Total isoprostanes were quantified in plasma (P-Isop) containing the preservatives BHT (0.005 %, w/v) and indomethacin (10 μM), which were added before storage. Hydrolysis of sterified isoprostanes and solid-phase extraction (using Sep-Pak Classic C18 and Sep Pak Vac-Silica columns, Waters® Corporation, Milford, MA, USA) were performed before the measurement of P-Isop by a competitive enzyme immunoassay (15-Isoprostane F2t ELISA Kit; Oxford Biomedical Research® Inc., Oxford, MI, USA), according to the protocol provided by the manufacturer. Plasma total antioxidant status (P-TAS) was evaluated by a spectrophotometric assay (Antioxidant Assay Kit; Cayman Chemical Company, Ann Arbor, MI, USA) that measures the combined antioxidant activities of water- and lipid soluble antioxidants, including vitamins, glutathione, uric acid, bilirubin, albumin, etc. The antioxidant capacity of the sample is compared with that of Trolox, a water-soluble tocopherol analogue, and is expressed as mM Trolox equivalents.

2.6. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) or as median (25th percentile; 75th percentile), or as percentage, and represented graphically as scatter plots. eGFR was determined using the Cockroft–Gault formula given its low cost and universal availability. [49]

Statistical analysis was performed using unpaired Student's *t*-test or Mann–Whitney U-test, where appropriate. Categorical variables were analysed by the Fisher's exact test. *P*-values of <0.05 were considered significant. For correlation analysis, a base 10 log transformation was applied to all variables with non-normal distribution. Pearson's single regression or Spearman's correlation analyses were used to estimate correlations between sets of parametric or nonparametric data, respectively. Multivariate linear regression was applied to determine the relationship between RAAS biomarkers and oxidative stress, between RAAS biomarkers and S-total 25(OH)D or between oxidative stress and S-total 25(OH)D, adjusted for some confounders namely age, gender and BMI. Only variables that presented significant correlations in Pearson's or Spearman's analyses were evaluated in the multivariate linear regression models. Coefficient regression (β), 95 % confidence intervals (95 % CI), *P*-value and coefficient of determination (R^2) were presented. The results were stratified according to renal function (eGFR <60 mL/ min per 1.73 m^2 or eGRF ≥ 60 mL/min per 1.73 m^2). There were missing values in some biomarkers due to insufficient sample volume of samples or reagents to perform sample processing, dilution tests and assays. The final number per group for these parameters was as following: troponin I, n = 34 vs 24 (mild-to-moderate CHF vs severe CHF) and n = 22 vs 36 (nRF vs iRF); urea, n = 35 vs 24 (mild-to-moderate CHF vs severe CHF) and n = 24 vs 35 (nRF vs iRF); proteinuria, n = 33 vs 22 (mild-to- moderate CHF vs severe CHF) and n = 22 vs 33 (nRF vs iRF); U-H_2O_2 and U-Isop, n = 35 vs 24 (mild-to-moderate CHF vs severe CHF); n = 23 vs 36 (nRF vs iRF); P-Isop, n = 33 vs 23 (mild-to-moderate CHF vs severe CHF); n = 20 vs 36 (nRF vs iRF); P-TAS, n = 33 vs 22 (mild-to-moderate CHF vs severe CHF) and n = 19 vs 35 (nRF vs iRF), P-renin, n = 30 vs 21 (mild-to-moderate CHF vs severe CHF) and n = 19 vs 32 (nRF vs iRF), P- Aldost, n = 33 vs 21 (mild-to-moderate CHF) and n = 21 vs 33 (nRF vs iRF); S-ACE, n = 29 vs 24 (mild-to-moderate CHF vs severe CHF) and n = 22 vs 31 (nRF vs iRF); P-AGT, n = 33 vs 20 (mild-to-moderate CHF vs severe CHF) and n = 20 vs 33 (nRF vs iRF); U-AGT, n = 35 vs 19 (mild- to-moderate CHF vs severe CHF) and n = 21 vs 32 (nRF vs iRF); P-Ang I, P-Ang II and P-Ang 1−7, n = 30 vs 13 (mild-to-moderate CHF vs severe CHF) and n = 17 vs 26 (nRF vs iRF); S-total 25(OH)D, n = 32 vs 21 (mild-to-moderate CHF vs severe CHF) and n = 20 vs 31 (nRF vs iRF).

Based on the values described by Chen et al. [19] for U-AGT values in patients with acute decompensated HF with or without progressed acute kidney injury, we found that a sample size of 19 patients in each group would be sufficient to obtain a statistical study power of 80 % at a 5% level of significance. We first used the formulas provided by Wan et al. [50] to estimate the sample mean and standard deviation

Table 1
Demographic, clinical and biochemical parameters of the study population stratified by CHF severity or by renal function.

Demographic, clinical and biochemical parameters	Mild-to-moderate CHF (n = 36)	Severe CHF (n = 24)	*P* value	CHF patients with normal renal function (nRF) (eGFR ≥ 60 mL/min per 1.73 m²) (n = 24)	CHF patients with impaired renal function (iRF) (eGFR < 60 mL/minper 1.73 m²) (n = 36)	*P* value
NYHA class I, n (%)	16 (44)	——	——	10 (42)	6 (17)	0.0410
NYHA class II, n (%)	20 (56)	——	——	9 (38)	11 (31)	0.5846
NYHA class III n (%)	——	7 (29)	——	2 (8)	5 (14)	0.6913
NYHA class IV, n (%)	——	17 (71)	——	3 (13)	14 (39)	0.0399
Age (years)	**67 ± 2**	**75 ± 2**	**0.0169**	**60 ± 2**	**76 ± 1**	**<0.0001**
Gender*: Men, n (%)	**27 (75)**	**11 (46)**	**0.0299**	19 (79)	19 (53)	0.0557
Gender*: Women, n (%)	**9 (25)**	**13 (54)**	**0.0299**	5 (21)	17 (47)	0.0557
Weight (kg)	**76 2**	**67 ± 2**	**0.0180**	**79 3**	**69 2**	**0.0047**
BMI (kg/m²)	1	26 ± 1	0.1153	**1**	**1**	**0.0417**
SBP (mmHg)	123 ± 3	114 ± 4	0.0990	119 ± 4	119 ± 4	0.9762
DBP (mmHg)	66 (60; 78)	68 (60; 70)	0.4724	68 (60; 78)	65 (60; 73)	0.3088
HR (beat/min)	72 ± 2	67 ± 2	0.0960	74 ± 3	68 ± 1	0.0638
Etiology						
Ischemic, n (%)	14 (39)	11 (46)	0.6056	**6 (25)**	**19 (53)**	**0.0375**
Hypertensive, n (%)	8 (22)	5 (21)	>0.9999	4 (17)	9 (25)	0.5336
Alcoholic cardiomyopathy, n(%)	6 (17)	4 (17)	>0.9999	**9 (38)**	**1 (3)**	**0.0007**
Valvular heart disease, n (%)	1 (3)	2 (8)	0.5746	1 (4)	2 (6)	>0.9999
Idiopatic cardiomyopathy/ Unknown, n (%)	7 (19)	3 (13)	0.3337	3 (13)	7 (19)	0.7255
Other, n (%)	1 (3)	1 (4)	>0.9999	2 (8)	0 (0)	0.1559
Treatment						
ACE inhibitor and/or ARB blocker, n (%)	**36 (100)**	**17 (71)**	**0.0009**	23 (96)	30 (83)	0.2254
Beta-blocker, n (%)	30 (83)	19 (79)	0.7412	19 (79)	30 (83)	0.7412
Loop diuretic, n (%)	32 (89)	24 (100)	0.1412	23 (96)	33 (92)	0.6434
Nitrates, n (%)	**3 (8)**	**8 (33)**	**0.0197**	**1 (4)**	**10 (28)**	**0.0375**
Biochemical parameters/ biomarkers						
BNP (pg/mL)	**215 (153;399)**	**822(337; 2013)**	**0.0003**	**185 (137;275)**	**504 (256;1685)**	**0.0002**
Troponin I (ng/mL)	**0.014 (0.005; 0.029)**	**0.032 (0.010;0.071)**	**0.0446**	0.011 (0.005;0.028)	0.025 (0.005;0.049)	0.1111
Urea (mmol/L) **eGFR** (mL/min per 1.73 m²).	**9.3 (6.8; 11.5)** **66.0 ± 4.6**	**12.9 (8.6; 19.5)** **45.3 ± 4.3**	**0.0112** **0.0027**	**8.5 (6.4; 10.1)** **83.8 ± 4.3**	**12.8 (8.8; 18.3)** **40.3 ± 2.0**	**0.0004** **<0.0001**
Proteinuria (g/day)	0.18 (0.15;0.22)	0.15 (0.11;0.21)	0.1360	0.17 (0.14;0.22)	0.17 (0.12;0.22)	0.5461
U-Na⁺ (mmol/mg creat.)	0.13 (0.10; 0.18) ± 28	0.17 (0.14;0.23)	0.0566	0.15 (0.11; 0.20) ± 29	0.14 (0.10;0.19) ± 26	0.3640

ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BMI, body mass index; BNP, B-type natriuretic peptide; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HR, heart rate; NYHA, New York Heart Association; SBP, systolic blood pressure; U-Na⁺, urinary excretion of sodium. Results are expressed as number (%), mean ± SEM or as median (25th percentile; 75th percentile) for data with normal or non-normal distribution, respectively.

Bold values: statistically significant results between groups.

from the sample size, median and 25th and 75thpercentiles values described by Chen et al. [19] and further calculated sample size using G*Power 3.1.

3. Results

3.1. Demographical, clinical and biochemical characteristics

Demographic and clinical characteristics of the study population are displayed in Table 1.

There were 36 patients with mild-to-moderate CHF (NYHA stages I and II) and 24 patients with severe CHF (NYHA stages III and IV) stratified by disease severity. Mild-to-moderate CHF patients were predominantly men and significantly younger and heavier than severe CHF patients. HF etiologies were similar among the groups, with ischaemic heart disease being the predominant cause of HF. Severe CHF group included significantly more patients receiving nitrates and less patients being treated with a RAAS inhibitor compared to the mild-to-moderate CHF group. No significant differences were observed for the other treatments. Severe CHF patients had significantly higher concentrations of BNP, troponin I, urea and lower values of eGFR.

When patients were stratified according to renal function, there were 24 patients with normal renal function (nRF, eGFR ≥ 60 mL/min per 1.73 m²) and 36 patients with impaired renal function (iRF, eGFR < 60 mL/min per 1.73 m²). Patients with iRF were significantly older and with lower weight and body mass index (BMI), had predominantly HF of ischemic etiology and consequently included a significantly higher number of patients treated with nitrates. They also presented higher BNP and urea concentrations. Alcoholic cardiomyopathy was the predominant cause of HF in nRF patients.

3.2. RAAS biomarkers

Patients stratified according to disease severity did not present significant differences in RAAS biomarkers, namely P-renin, P-Aldost, S- ACE, P-AGT, U-AGT, P-Ang I, P-Ang II or P-Ang 1− 7 (Table 2). However, when patients were stratified by renal function, those with iRF had significantly higher U-AGT values (more than a two-fold increase in median values, p = 0.003) than patients with nRF (Table 2).

3.3. Oxidative stress biomarkers

Patients with severe CHF had significantly higher U-Isop ($p = 0.002$) than mild-to-moderate CHF patients, but there were no significant differences for the other oxidative stress biomarkers. When patients were stratified by renal function, we did not observe differences in oxidative stress biomarkers between groups (Table 2).

3.4. Vitamin D status

Severe CHF patients had significantly lower values of S-total 25(OH) D compared to patients with mild-to-moderate CHF ($p = 0.006$). In patients stratified by renal function, those with iRF also presented significantly reduced S-total 25(OH)D compared to nRF patients ($p = 0.028$) (Table 2).

3.5. Correlation analysis

Patients with mild-to-moderate CHF exhibited significant inverse correlations between oxidative stress and RAAS markers (Log U-H_2O_2 vs P-Ang II; Log P-Isop vs Log P-AGT) (Fig. 1A and B, respectively), although there was a positive correlation of Log U-Isop with Log S-ACE (Fig. 1C). On the other hand, severe CHF patients only exhibited a significant positive correlation between Log U-H_2O_2 and Log P-renin (Fig. 1D). No correlation was observed between Log P-AGT and Log U- AGT.

When patients were stratified by renal function, the group with nRF presented inverse correlations between oxidative stress and RAAS markers, namely Log U-H_2O_2 vs P-Ang II (Fig. 2B) and Log P-Isop vs Log P-AGT) (Fig. 2C), as observed in the mild-to-moderate CHF group

Table 2
Oxidative stress, RAAS biomarkers and vitamin D status in the study population stratified by CHF severity or by renal function.

Oxidative stress and RAAS biomarkers	Mild-to-moderate CHF	Severe CHF	*P* value	CHF patients with normal renal function (nRF) (eGFR ≥ 60 mL/min per 1.73 m^2)	CHF patients with impaired renal function (iRF) (eGFR < 60 mL/min/ per 1.73 m^2)	*P* value
U-H_2O_2 (μmol/mg creat.)	0.009 (0.005;0.020)	0.012 (0.006;0.023)	0.483	0.011 (0.005;0.020)	0.011 (0.005;0.022)	>0.999
U-Isop (ng/mg creat.)	**2.2 (1.5;2.7)**	**3.2 (2.3;4.2)**	**0.002**	2.4 (1.8; 3.2)	2.6 (1.7; 3.5)	0.519
P-Isop (ng/mL)	0.6 (0.4;0.8)	0.8 (0.5;1.1)	0.065	0.7 (0.4;1.0)	0.6 (0.5;1.0)	0.803
P-TAS (mmol Trolox equiv./L)	2.4 (1.5;3.3)	1.5 (1.3;3.6)	0.873	2.2 (1.4; 3.4)	2.1 (1.4; 3.4)	0.918
P-AGT (μg/mL)	24 (19;29)	26 (19;36)	0.272	24 (16;35)	26 (20;30)	0.309
U-AGT (μg/g creat.)	16 (9;39)	29 (9;39)	0.502	**13 (7;20)**	**30 (13;64)**	**0.003**
P-Renin (μU/mL)	146 (29;260)	150 (56;682)	0.266	143 (64; 287)	151 (49;271)	0.900
P-Aldost (ng/dL)	9 (4;15)	14 (7;20)	0.131	9 (6;16)	12 (6;17)	0.789
S-ACE (U/L)	4 (2;7)	7 (2;33)	0.132	6 (4;10)	3 (2;22)	0.062
P-Ang I (pg/mL)	46.6 ± 2.1	46.8 ± 5.2	0.974	46.7 ± 2.2	46.6 ± 3.3	0.978
P-Ang II (pg/mL)	41.0 ± 2.9	35.9 ± 5.3	0.361	43.1 ± 4.3	37.0 ± 3.2	0.225
P-Ang 1¡7 (pg/mL)	27.2 ± 3.5	33.1 ± 4.2	0.325	28.2 ± 4.2	29.5 ± 3.7	0.819
S-total 25 (OH) D (ng/mL)	**18 (11;24)**	**11 (6;17)**	**0.006**	**18 (12;25)**	**11 (6;18)**	**0.028**

P-AGT, plasma angiotensinogen; P-Aldost, plasma aldosterone; P-Ang I, plasma angiotensin I; P-Ang II, angiotensin II; P-Ang (1-7), plasma angiotensin 1-7; P-Isop, plasma isoprostanes; P-renin, plasma renin concentration; P-TAS, plasma total antioxidant status; S-ACE, serum angiotensin-converting enzyme activity; S-total 25 (OH)D, serum total 25-hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-H_2O_2, urinary hydrogen peroxide; U-Isop, urinary isoprostanes. Results are expressed as mean ± SEM or as median (25th percentile; 75th percentile) for data with normal or non-normal distribution, respectively. Bold values: statistically significant results between groups.

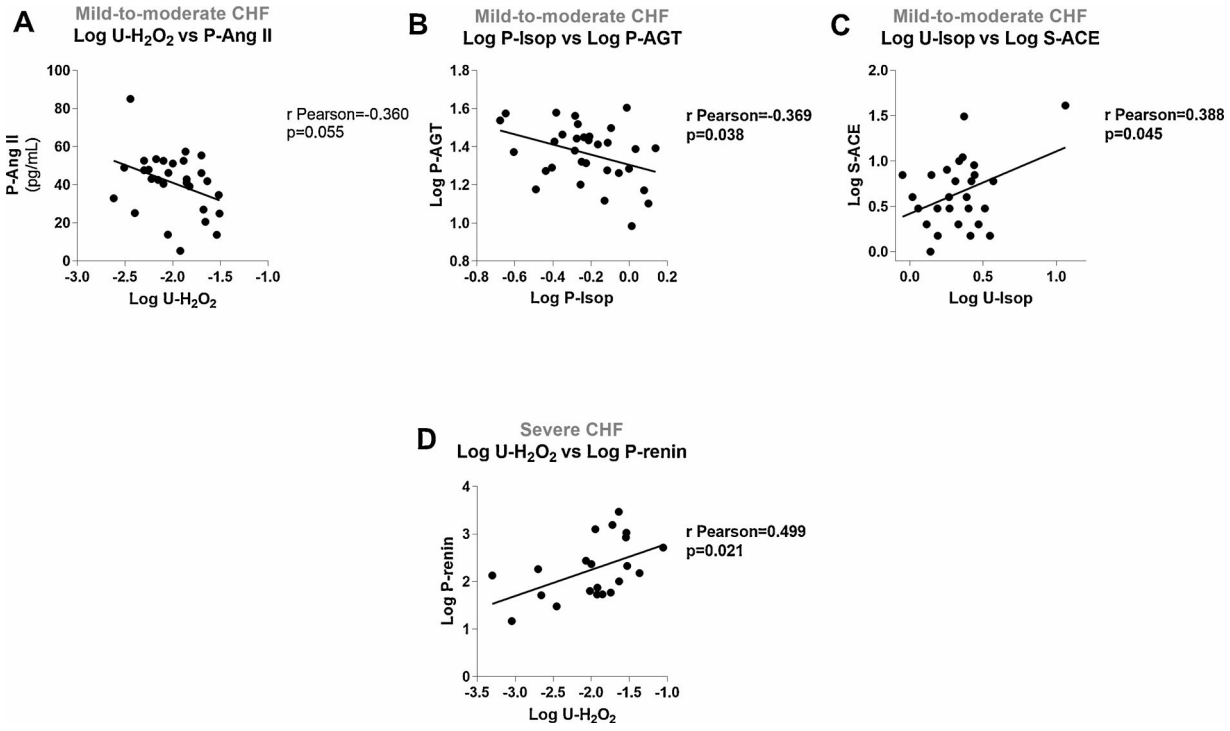


Fig. 1. Correlations between oxidative stress markers and RAAS parameters in mild-to-moderate and severe CHF: **(A)** Log U-H_2O_2 vs P-Ang II, mild-to-moderate CHF; **(B)** Log P-Isop vs Log P-AGT, mild-to-moderate CHF; **(C)** Log U-Isop vs Log S-ACE, mild-to-moderate CHF; (D) Log U-H_2O_2 vs Log P-renin, severe CHF.

(Fig. 1), but a positive correlation of Log U-H_2O_2 with P-Ang I (Fig. 2A). Differently, patients with iRF exhibited several significant positive correlations between oxidative stress and RAAS biomarkers, namely Log U-H_2O_2 vs Log P-renin (Fig. 3A), Log U-H_2O_2 vs Log P-AGT (Fig. 3B), Log U-Isop vs Log P-AGT (Fig. 3D) and Log U-Isop vs Log S-ACE (Fig. 3E) and a borderline significant positive correlation between Log U-H_2O_2 and Log U-AGT (p = 0.057) (Fig.3C). No correlation was observed between Log P-AGT and Log U-AGT.

In an attempt to identify factors that could be contributing to these correlations in CHF patients with nRF or with iRF, we also evaluated the correlations of S-total 25(OH)D with RAAS or oxidative stress biomarkers. We observed that S-total 25(OH)D values were positively correlated with P-Ang I in CHF-nRF patients (Fig. 4A), but not in CHF- iRF (Fig. 4B), and inversely correlated with U-Isop in CHF patients with iRF (Fig. 4D), but not in CHF-nRF (Fig. 4C).

3.6. Multivariate regression models

In the multivariate regression models adjusted for age, gender and BMI and stratified for eGFR, we observed that, in CHF patients with nRF, correlations were primarily inverse. Also, we found higher concentration of U-AGT and lower values of serum total 25(OH)D in CHF patients with iRF compared to those with nRF.

U-AGT has been considered a biomarker of intrarenal RAAS modulation since AGT is locally produced in the S3 segment of the proximal tubule in higher extent than in the liver [51], and U-AGT levels are associated with increases in AGT expression and Ang II concentration in the kidney [17,52]. Concurrently, there is limited glomerular permeability to liver-derived plasma AGT and this has a minor contribution to U-AGT levels since it is mostly up taken from the urinary filtrate by megalin [51]. Under pathological conditions like severe podocyte injury, glomerular filtration of AGT of liver origin is increased by the loss of the macromolecular barrier function of the glomeruli [53]. Nevertheless, it has also been demonstrated that increased U-AGT precedes the onset of albuminuria in diabetic patients and is present in normo-albuminuric children with diabetes [54,55]. Also, in patients with chronic kidney disease, U-AGT values are increased and negatively associated with eGFR, independently of urinary albumin excretion [56]. Our CHF patients had similar levels of proteinuria, even when we stratified patients according to the presence of renal impairment. Furthermore, there were no

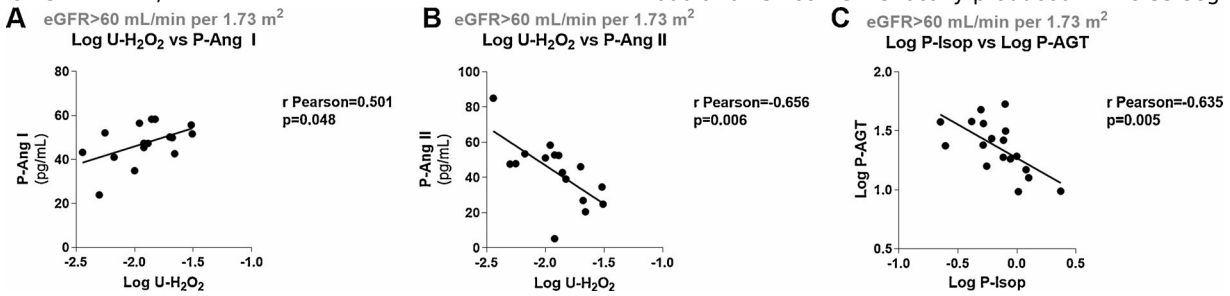


Fig. 2. Correlations between oxidative stress markers and RAAS parameters in CHF patients with nRF (eGFR >60 mL/min per 1.73 m^2): **(A)** Log U-H_2O_2 vs P-Ang I; **(B)** Log U-H_2O_2 vs P-Ang II; **(C)** Log P-Isop vs Log P-AGT.

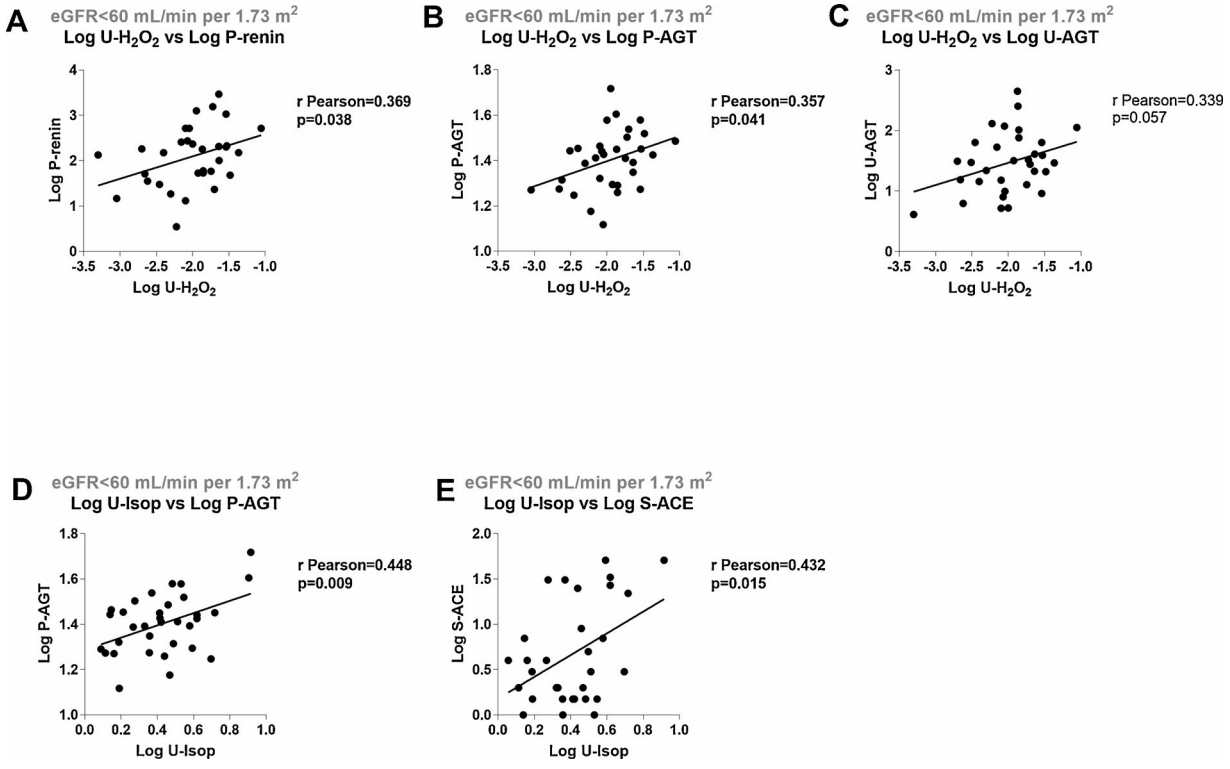


Fig. 3. Correlations between oxidative stress markers and RAAS parameters in CHF patients with iRF (eGFR < 60 mL/min per 1.73 m^2): **(A)** Log U-H_2O_2 vs P-renin; **(B)** Log U-H_2O_2 vs Log P-AGT; **(C)** Log U-H_2O_2 vs Log U-AGT; **(D)** Log U-Isop vs Log P-AGT; **(E)** Log U-Isop vs Log S-ACE.

the relationships between RAAS, oxidative stress and vitamin D status found in correlation analyses did not hold, although a borderline significant inverse association was shown for U-H_2O_2 and P-Ang II ($p = 0.068$) (Table 3).

In CHF patients with iRF, the relationships detected between U-Isop and RAAS markers or between vitamin D and U-Isop were confirmed in multivariate analysis, with U-Isop being associated with higher P-AGT ($R^2 = 0.46$) and S-ACE values ($R^2 = 0.54$), and S-total 25(OH)D being associated with lower U-Isop ($R^2 = 0.38$) (Table 4).

4. Discussion

Our study highlights the existence of several positive correlations between oxidative stress and RAAS biomarkers in CHF patients with iRF, while in CHF patients with nRF the

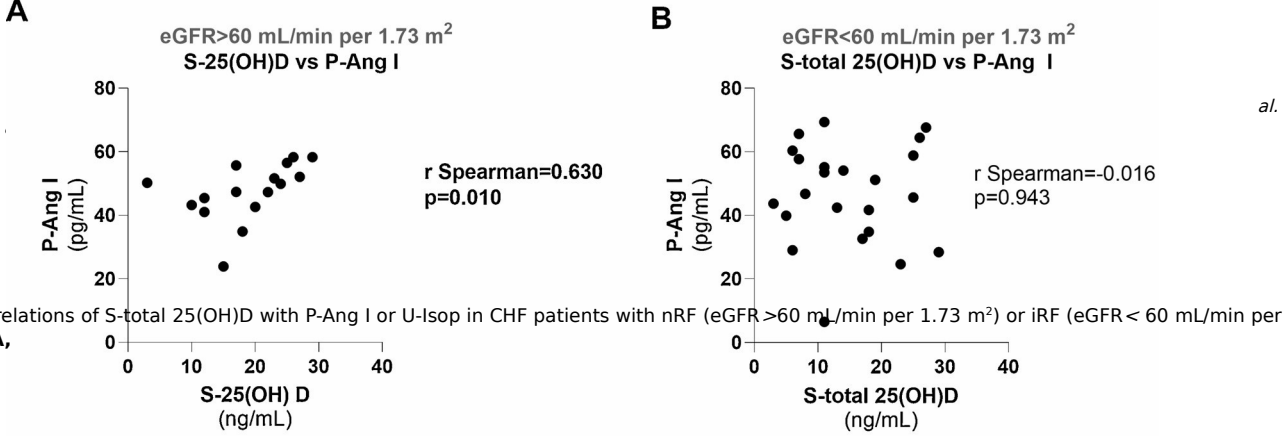
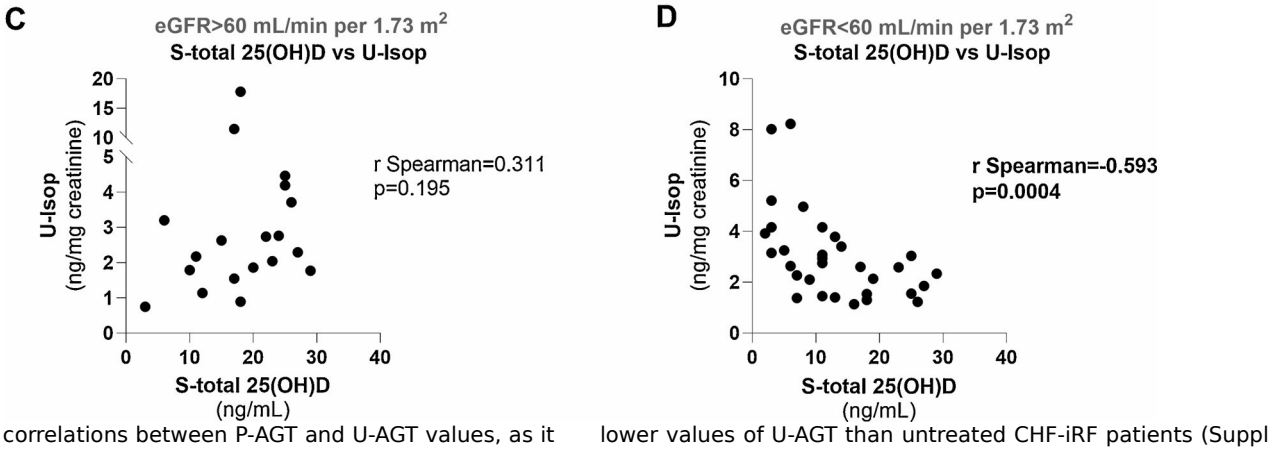


Fig. 4. Correlations of S-total 25(OH)D with P-Ang I or U-Isop in CHF patients with nRF (eGFR >60 mL/min per 1.73 m^2) or iRF (eGFR < 60 mL/min per 1.73 m^2): **(A, B)** S-total 25(OH)D vs P-Ang-I; **(C, D)** S-total 25(OH)D vs U-Isop.



significant correlations between P-AGT and U-AGT values, as it would be expected if U-AGT values resulted from filtration of P-AGT. Therefore, the increased U-AGT values observed in our study appear to mainly reflect intrarenal RAAS activation in CHF patients with iRF. U-AGT has been recently demonstrated to be an early predictor for acute CRS and 1-year prognosis in patients with acute decompensated HF [57]. When measured at the time of acute kidney injury diagnosis (AKI), U-AGT was also useful to predict AKI progression and to identify CRS patients with higher risk for adverse outcomes [19]. Thus, U-AGT appears to be an important biomarker for risk stratification in HF.

In our study, the lack of significant differences in other ROS and RAAS parameters between CHF patients with or without renal impairment, besides those observed in U-AGT, might be due to treatment with RAAS blockers and nitrates, which are known to influence both oxidative stress and RAAS markers [58–61].

The positive relationships found between urinary markers of oxidative stress and systemic RAAS parameters in CHF-iRF patients are not surprising. U-AGT is considered a marker of intrarenal AGT production [17,51,52], but U-H_2O_2 and U-Isop can reflect both systemic and renal oxidative stress [39,62–66] and systemic biomarkers of oxidative stress have been shown to correlate or associate with systemic RAAS [67–69]. Of note, a borderline significant positive correlation between U-H_2O_2 and U-AGT was also detected in patients with renal function impairment. This could mean that higher intrarenal H_2O_2 might be associated with intrarenal RAAS activation in these patients. The lack of a stronger correlation between these two biomarkers could be due to the small number of patients evaluated and to the fact that U-H_2O_2 does not solely reflect renal H_2O_2 production. Furthermore, some patients of this group were receiving nitrates which might decrease intrarenal RAAS activation and exert a protective effect on cardiorenal disease [58,61]. Interestingly, we observed that CHF-iRF patients treated with nitrates exhibited tendentially lower values of U-AGT than untreated CHF-iRF patients (Suppl Fig. 1H).

Experimental studies have shown that increased ROS bioavailability enhances RAAS activation, contributing to the development and progression of renal disease. In a model of diabetic nephropathy in Zucker diabetic fatty obese rats, an increase in U-Isop precedes the rise of renal AGT and Ang II concentrations, as well as of renal injury markers [70]. Also, in a mice model of IgA nephropathy, both ROS and RAAS activation contribute to renal injury in an early phase of the disease [71]. Furthermore, treatments with antioxidants have been shown to reduce the renal expression or urinary excretion of AGT in experimental models of arterial hypertension [23,72]. Noteworthy, our present results show, for the first time, that oxidative stress and RAAS biomarkers are positively correlated in CHF patients with renal impairment, while in CHF patients with nRF there were mainly inverse correlations between ROS and RAAS. This suggests that a positive feedback mechanism between ROS and RAAS, or vice-versa, operates in CRS, despite medical therapy, while in CHF patients with normal renal function, an effective negative feedback regulation between RAAS and ROS might be relevant to preclude renal impairment. Interestingly, we observed that, in patients with normal renal function, but not in patients with renal dysfunction, Ang II exhibited a borderline positive correlation with Ang 1-7 (Suppl Fig), which is known to counterregulate Ang II-induced ROS generation [73, 74]. Thus, we speculate that Ang 1-7 production from Ang II might be an important factor contributing to the inverse relationships observed in CHF patients with normal renal function. Nevertheless, we cannot exclude other counterregulatory mechanisms beyond Ang 1− 7 production, such as an inhibitory effect of H_2O_2 (or other ROS or ROS by-products) on the expression and/or activity of Ang II-producing enzymes [75,76], or a stimulatory effect on Ang II-degrading enzymes. In fact, ROS have been shown to either upregulate or downregulate the

Table 3
Multivariate linear regression models for RAAS markers (P-Ang I, P-Ang II and P- AGT) in CHF patients with eGFR≥60 mL/min per 1.73 m^2.

(Coefficient regression (Adjusted β), 95 % confidence intervals (95 % CI), *P* value and coefficient of determination (R^2) estimated by linear regression models with P-Ang I, P-Ang II and P-AGT as the dependent variable and adjusted for age, gender and BMI).

eGFR≥ 60 mL/min per 1.73 m				
	Adjusted β	95 % CI		*P* value
R^2				
P-Ang I (pg/mL)				
Age (Years)	− 0.105	− 0.703	0.493	0.701
Gender (Male)	− 8.834	−	15.306	0.429
BMI (kg/m²)	0.719	32.973	2.034	
		− 0.596		0.247
U-H_2O_2 (μmol/mg creat)	312.356	− 500.019	1124.731	0.407
R_2	0.54			
P-Ang II (pg/mL)				
Age (Years)	0.438	− 0.409	1.284	0.272
Gender (Male)	− 4.495	−	29.675	0.773
BMI (Kg/m²)	0.665	38.665	2.527	
		− 1.196		0.440
U-H_2O_2 (μmol/mg creat)	− 1055.904	− 2205.825	94.016	0.068
R_2	0.50			
P-AGT (μg/mL)				
Age (Years)	0.387	− 0.528	1.302	0.358
Gender (Male)	− 6.922	−	14.146	0.470
BMI (kg/m²)	0.340	27.991	2.161	
		− 1.481		0.678
P-Isop (ng/mL)	− 25.999	− 57.414	5.415	0.093
R_2	0.43			
P-Ang I (pg/mL)				
Age (Years)	− 0.103	− 0.961	0.754	0.791
Gender (Male)	− 6.440	−	21.711	0.617
BMI (kg/m²)	0.855	34.592	2.669	
		− 0.958		0.314
S-total 25(OH)D (ng/mL)	0.108	− 1.304	1.519	0.867
R_2	0.31			

BMI, body mass index, P-AGT, plasma angiotensinogen; P-Ang I, angiotensin I; P-Ang II, plasma angiotensin II; P-Isop, plasma isoprostanes; S-total 25(OH)D, serum total 25-hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-Isop, urinary isoprostanes.

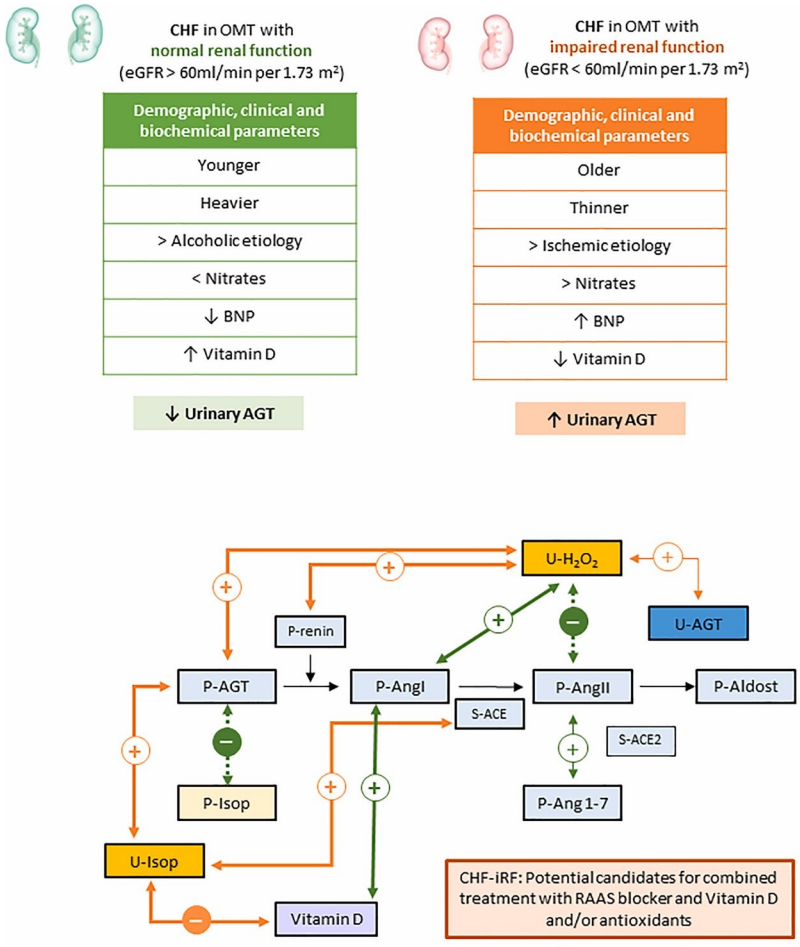
expression and activity of RAAS parameters, depending on the physiological or pathological condition, tissues and species tested [28].

Renal disease is often associated with vitamin D insufficiency or deficiency [40,41]. In our study, CHF patients with impaired renal function had significantly lower values of 25(OH)D, a biomarker of vitamin D status, compared to patients with preserved renal function. Since vitamin D has been shown to reduce RAAS activation and oxidative stress [42–45], we investigated the correlations of serum 25(OH)D with RAAS and oxidative stress parameters in patients with or without renal impairment. Importantly, we observed that 25(OH)D was inversely correlated with U-Isop only in CHF-iRF patients and positively correlated with P-Ang I only in CHF-nRF. Furthermore, the inverse relationship between 25(OH)D and U-Isop, as well as the positive associations of U-Isop with P-AGT or S-ACE in CHF-iRF patients held in multivariate analysis controlled for age, gender and BMI. This suggests that markedly low vitamin D values in CHF-iRF patients contribute to enhanced oxidative stress that further aggravates RAAS activation, despite RAAS blockade. Indeed, a growing amount of experimental evidence and a recent meta-analysis of clinical trials have shown that vitamin D supplementation prevents or attenuates oxidative stress and associated damage through multiple mechanisms [42,45]. Therefore, the combination of RAAS blockers with vitamin D and/or antioxidants might be useful to block these escape mechanisms and prevent further cardiac and renal deterioration in CHF patients with renal dysfunction. Of note, vitamin D supplementation was shown to prevent the decline in eGFR in CKD patients using RAAS blockers [77]. Also, more recently, the authors of a study in coronary artery disease patients undergoing percutaneous coronary intervention underlined the need of conducting larger studies to assess the effect of vitamin D supplementation on RAAS

Table 4

Multivariate linear regression models for RAAS (P-Renin, P-AGT, U-AGT, S-ACE) and oxidative stress (U-Isop) markers in CHF patients with eGFR<60 mL/min per 1.73m². (Coefficient regression (Adjusted β), 95 % confidence intervals (95 % CI), *P* value and coefficient of determination (R^2) estimated by linear regression models with P-Renin, P-AGT, U-AGT, S-ACE or U-Isop as the dependent variable and adjusted for age, gender and BMI.).

eGFR<60 mL/min per 1.73 m				
	Adjusted β	95 % CI		*P* value
R^2				
P-Renin (μU/mL)				
Age (Years)	− 10.544	− 50.942	29.855	0.592
Gender (Male)	397.046	− 179.969	974.060	0.167
BMI (kg/m2)	− 9.537	− 77.903	58.828	0.774
U-H_2O_2 (μmol/mg creat)	4494.721	− 11804.430	20793.872	0.572
R_2	0.15			
P-AGT (μg/mL)				
Age (Years)	− 0.466	− 0.988	0.056	0.077
Gender (Male)	− 1.287	−	6.067	0.719
BMI (kg/m²)	0.200	8.640	1.051	0.629
		− 0.651		
U-H_2O_2 (μmol/mg creat)	137.674	− 69.067	344.416	0.180
R_2	0.21			
U-AGT (μg/g creat)				
Age (Years)	− 0.002	− 2.315	2.311	0.999
Gender (Male)	17.285	−	50.646	0.292
BMI (kg/m²)	− 2.417	16.075	1.505	0.213
		− 6.339		
U-H_2O_2 (μmol/mg creat)	407.749	− 506.935	1322.432	0.363
R_2	0.19			
P-AGT (μg/mL)				
Age (Years)	− 0.399	− 0.831	0.034	0.069
Gender (Male)	0.769	−	6.995	0.799
BMI (kg/m²)	0.249	5.457	0.933	0.456
		− 0.435		
U-Isop (ng/mg creat)	**3.260**	**1.307**	**5.214**	**0.002**
R_2	0.46			
S-ACE (U/L)				
Age (Years)	− 0.150	− 0.777	0.477	0.624
Gender (Male)	3.685	− 4.879	12.248	0.381
BMI (kg/m²)	**1.242**	**0.253**	**2.231**	**0.016**
U-Isop (ng/mg creat)	**6.063**	**3.257**	**8.870**	**<0.001**
R_2	0.54			
U-Isop (ng/mg creat)				
Age (Years)	− 0.066	− 0.160	0.028	0.159
Gender (Male)	0.274	− 1.228	1.777	0.705
BMI (kg/m²)	− 0.019	− 0.189	0.150	0.812
S-total 25(OH)D (ng/mL)	**¡0.133**	**¡0.233**	**¡0.033**	**0.012**
R^2	0.38			



CHF-nRF: ⊖ **negative feedback regulation of RAAS:**
The inverse correlation between H_2O_2 and P-Ang II might be due to increased formation of P-Ang 1-7 from Ang II, since Ang 1-7 might counterregulate Ang II-induced ROS generation.
The positive correlation between H_2O_2 and Ang I might result, at least in part, from an inhibitory effect of H_2O_2 on ACE activity; in addition, H_2O_2 could also have a stimulant effect on Ang II-degrading enzymes, such as ACE2, aminopeptidases, endopeptidases, carboxipeptidases and neprilysin, which would contribute to the increase in P-Ang 1-7 levels; ROS or ROS-by products (isoprostanes) could also inhibit systemic AGT expression; Vitamin D increases Ang I probably by inhibiting ACE or other Ang II-forming enzymes.

CHF-iRF: ⊕ **positive feedback regulation of RAAS:**
Low Vitamin D values contribute to enhanced oxidative stress; ROS (H_2O_2) and ROS by-products (isoprostanes) increase the expression and/or activity of systemic RAAS components (AGT, renin, ACE) and intra-renal RAAS (U-AGT).

BMI, body mass index; P-AGT, plasma angiotensinogen; P-Ang I, plasma angiotensin I; P-Ang II, plasma angiotensin II; P-Isop, plasma isoprostanes; P- Renin, plasma renin; S-ACE, serum angiotensin-converting enzyme activity; S- total 25(OH)D, serum total 25-hydroxyvitamin D; U-AGT, urinary angiotensinogen; U-Isop, urinary isoprostanes.
Bold values: statistically significant results between groups.

modulation, principally in those patients treated with RAAS inhibitors [78]. Additionally, a positive impact of antioxidant treatment on cardiovascular morbidity and mortality was already shown in patients with end-stage renal disease, while no benefits were observed in high cardiovascular risk patients without renal disease [29,30].

A major strength of our study is that we evaluated HF patients in "real-world conditions", who, as emphasized by DeVore et al., for most health systems, represent the vast majority of patients, in opposition to the small percent enrolled in clinical trials [79]. Of note, there is a lack of unambiguous evidence of clinical efficacy for many HF guideline-directed medical therapy (GDMT) in patients not adequately represented in HF trials [80]. Moreover, in advanced HF stages, some of the recommendations with proven benefits in earlier stages of HF are no longer effective [80]. Our patients presented different HF etiologies and comorbidities, and were on polypharmacy with drugs that are known to interfere with both RAAS and oxidative stress markers (RAAS blockers, beta-blockers, diuretics, nitrates). Although this might also be considered a limitation, we think that this study pushes forward the comprehension of the "real-world impact" on RAAS and ROS in HF patients, suggesting personalized therapeutic approaches to fulfil the still-unmet needs in HF management and prognosis.

A schematic representation of the interplay between RAAAS and oxidative stress markers in CHF patients with or without renal impairment and on optimized medical therapy is shown in Fig. 5.

5. Conclusions

CHF patients with iRF have higher intrarenal RAAS activation and lower serum vitamin D values, exhibiting several positive associations between oxidative stress and RAAS markers, in contrast to CHF patients with nRF. The inverse association of vitamin D with U-Isop, which in turn is positively associated with RAAS markers in CHF patients with renal impairment, suggests that, in these patients, severe vitamin D deficiency-driven oxidative stress limit RAAS blockers efficacy by promoting RAAS upregulation. Furthermore, our results suggest that stratifying CHF patients by renal function may be better than stratifying by functional class to identify those with intrarenal RAAS activation, which will probably benefit from the combination of RAAS blockers with vitamin D and/or antioxidants [5,28,81,82].

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Fig. 5. Schematic representation of the interplay between RAAS and oxidative stress markers in CHF patients with normal (nRF, green) or impaired (iRF, orange) and on optimized medical therapy (OMT).
AGT, angiotensinogen; **BNP**, B-type natriuretic peptide; **CHF**, chronic heart failure; **eGFR**, estimated glomerular filtration rate; **OMT**, optimized medical therapy; **P-AGT**, plasma angiotensinogen; **P-Ang I**, plasma angiotensin I; **P-Ang II**, plasma angiotensin II; **P- Ang 1¡7**, plasma angiotensin 1− 7; **S-ACE**, serum angiotensin- converting enzyme; **S-ACE2**, serum angiotensin-converting enzyme type 2; **P-Isop**, plasma isoprostanes; **P-renin**, plasma renin; **RAAS**, renin-angiotensin-aldosterone system; **P-Aldost**, plasma aldosterone; **U-AGT**, urinary angiotensinogen; **U-Isop**, urinary isoprostanes; **U-H_2O_2**,

urinary hydrogen peroxide. **Green filled arrows**, positive correlations in CHF-nRF patients; **green dotted arrows**, negative correlations in CHF-nRF patients; **orange arrows**, positive correlations in CHF-iRF patients. **Blue boxes**, RAAS markers (light blue for plasma, dark blue for urine); **yellow boxes**, oxidative stress markers (light yellow for plasma, dark yellow for urine).

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110938.

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